J. Parasitol., 89(4), 2003, pp. 851–853

Toxoplasma gondii Isolates of Free-Ranging Chickens From Rio de Janeiro, Brazil: Mouse Mortality, Genotype, and Oocyst Shedding by Cats

J. P. Dubey, D. H. Graham*, Daniele Seipel da Silva†, T. Lehmann*, and Lilian M. G. Bahia-Oliveira†, Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, United States Department of Agriculture, Agricultural Research Service, Building 1001, Beltsville, Maryland 20705; *Division of Parasitic Diseases, Centers for Disease Control and Prevention, 4770 Bufford Highway, MS: F22, Chamblee, Georgia 30341; †Laboratório de Biologia do Reconhecer, Centro de Biociências e Biotecnologias, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes 28015, Brazil. *e-mail: jdubey@anri.barc.usda.gov*

ABSTRACT: Most isolates of *Toxoplasma gondii* can be grouped into 3 genetic lineages. In the present study, 67 isolates of *T. gondii* were obtained by bioassay in mice inoculated with brains and hearts of 96 asymptomatic chickens from an area highly endemic to human infection in Rio de Janeiro, Brazil. Of the 48 isolates genotyped using the SAG_2 locus, 34 (70%) were of type I and 13 (27%) were of type III. No isolate of type II was recovered. Isolates from 1 chicken contained a type I and type III mixed infection, indicating natural multiparasite infection in the same animal. Cats fed mice infected with 11 type I strains shed 19–535 million oocysts in their feces, indicating that type I isolates can circulate in the environment.

Toxoplasma gondii infections are widely prevalent in human beings and animals worldwide (Dubey and Beattie, 1988). Humans become infected postnatally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or accidentally ingesting oocysts in the environment. However, only a small percentage of exposed adult humans develop clinical signs. It is not known whether the severity of toxoplasmosis in immunocompetent persons is due to the parasite strain, host variability, or other factors.

Overall, there is low genetic diversity among the *T. gondii* isolates examined so far. *Toxoplasma gondii* isolates have been classified into 3 genetic types (I, II, and III) based on restriction fragment length polymorphism (RFLP) (Howe and Sibley, 1995; Howe et al., 1997). It has been suggested that type I strains or recombinants of types I and III are more likely to result in clinical ocular toxoplasmosis (Grigg, Gantara et al., 2001), but genetic characterization has been limited essentially to isolates from patients ill with toxoplasmosis. In contrast to the situation in humans, most of the genetically typed isolates of *T. gondii* have come from animals, and most of these isolates were of type II or type III (Howe and Sibley, 1995; Mondragon et al., 1998; Owen and Trees, 1999; Jungersen et al., 2002). *Toxoplasma gondii* isolates differ markedly in their virulence to outbred mice. Type I isolates are more virulent to mice than types II and III isolates, and the virulence is genetically controlled (Howe et al., 1996; Grigg, Bonnefoy et al., 2001; Su et al., 2002). Because type I isolates do not give rise to chronic infections in mice, it has been speculated that such isolates may not produce oocysts in cats and may even belong to a separate species of *Toxoplasma* (Dardé, 1996; Johnson, 1997).

Recently, we found that 17 of 25 isolates of *T. gondii* obtained from asymptomatic free-range chickens from rural areas surrounding São Paulo, Brazil, were of type I (Dubey et al., 2002). Because chickens become infected mostly by feeding from soil contaminated with oocysts, prevalence of *T. gondii* in chickens is a good indicator of the strains prevalent in their environment (Ruiz and Frenkel, 1980). In the present article, we confirm these findings using free-range chickens from another area of Brazil (Rio de Janeiro), geographically separated from São Paulo, and examine these isolates with respect to mouse mortality, genotype, and oocyst shedding by cats.

Materials and methods were essentially the same as those reported previously (Dubey et al., 2002). Brains and hearts of 86 chickens from Campos dos Goytacazes (Campos) (Silva et al., 2003), and 10 additional chickens from other areas near Campos were bioassayed in outbred female Swiss Wester mice obtained from Taconic Farms, Germantown, New York. Briefly, the brain and heart of each chicken were pooled, homogenized, digested in acidic pepsin, and washed; the homogenate was then inoculated s.c. into 5 mice. Tissue imprints of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 35 PI and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the modified agglutination test (MAT) (Dubey and Desmonts, 1987). Mice were killed 40 days PI, and their brains were examined for tissue cysts as described (Dubey and Beattie, 1988).

For mice that died within 4 wk PI, lungs or brain were removed from the inoculated mice. A portion of tissue was frozen at −70 C for deoxyribonucleic acid (DNA) characterization. The remaining tissue was homogenized in saline for subinoculation into 2 mice. Subinoculated mice that were given strains of *T. gondii* that previously killed mice within 3 wk PI were given sulfadiazine sodium (1 mg/ml) from days 7 to 20 PI. Brains of those mice that survived for 3 wk PI were examined for tissue cysts. Tissues from mice inoculated with 13 of these mouse virulent isolates of *T. gondii* were selected to obtain oocysts (Table I). For this, brains of 1 or 2 mice inoculated with murine tissues 17–32 days previously were fed to 13 cats (Table I). Feces of cats were examined for shedding of *T. gondii* oocysts as described previously (Dubey, 1995). The total number of oocysts shed by each cat was counted as described (Dubey et al., 2002). To determine the parasite genotypes of oocysts of these 13 isolates, oocysts were sporulated and bioassayed in mice (Dubey and Beattie, 1988). Four days after being fed oocysts, mice that died, or those that were killed, were necropsied, and their mesenteric lymph nodes were removed for *T. gondii* DNA isolation.

Toxoplasma gondii DNA was extracted from mouse tissue as described previously (Lehmann et al., 2000). The RFLP genetic type of 48 randomly selected isolates was determined by nested polymerase chain reaction (PCR) on the SAG_2 locus according to Howe et al. (1997).

In total, there were 67 isolates of *T. gondii* from chickens from the Rio de Janeiro area. Sixty-one isolates were the same as those obtained by Silva et al. (2003). The remaining 6 isolates were from 10 chickens with MAT titers of $<$1:10 in 1 chicken, 1:25 in 2, and 1:100 or more in 3 chickens.

The number of *T. gondii*–positive mice of the 5 inoculated varied. In 47 isolates, 5 mice were infected; in 9 isolates, 4 of 5 mice were infected; in 3 isolates, 3 mice were infected; in 3 isolates, 2 mice were infected; and in 5 isolates only 1 mouse was infected.

Of the 48 *T. gondii* isolates genotyped, 34 (70%) were type I, 13 (27%) were type III, and 1 was mixed with types I and III. With 33 of 34 type I isolates, 100% of infected mice died of toxoplasmosis between 8 and 28 days. In the case of the 34th isolate, 2 mice died 19 or 26 days PI, and 3 mice survived until they were killed 40 day PI; *T. gondii* DNA from only 1 of these 5 mice was genotyped, and this mouse had survived. Of the 13 type III isolates examined, mouse mortality varied from 0 (5 isolates) to 100% (4 isolates) and 20–80% (4 isolates). Type I and type III isolates were recovered from 1 chicken (no. 230). One of the 5 mice inoculated with tissues of chicken no. 230 died day 31 PI, and it was found to have been infected with type I; the remaining 4 mice that survived were killed on day 40, and 1 of these was type I and 3 were of type III.

Mouse mortality with different isolates varied. In 50 isolates, all infected mice died of toxoplasmosis. The percent mortality and the number of remaining 17 isolates were 80 (1), 75 (1), 60 (1), 50 (3), 40 (2), 20 (2), and 0 (7).

The number of *T. gondii* in tissues of food animals is low as indicated by bioassays (Dubey et al., 1995). Therefore, for bioassays in mice, tissues of food animals are digested with pepsin or trypsin to release bradyzoites from tissue cysts before inoculation into mice. During pro-

TABLE I. *Toxoplasma gondii* oocyst shedding by cats fed mice infected with isolates from chickens from Rio de Janeiro, Brazil.

Chicken no.	Cat no.	Day of infection in mice*	No. of mice fed	No. of oocysts shed ($\times 10^6$)	*T. gondii* genotype†
211	IOB1	26 (D)	1	194	I
192	699	27 (D)	1	95	I
70	700	23 (D)	2	19	I
68	102	26 (K)	2	276	I
57	671	27 (K)	1	280	I
29	672	28 (D)	1	130	I
229	673	32 (K)	1	39	I
229	674	32 (K)	1	22	I
14	377	32 (K)	1	209	I
205	379	28 (K)	1	21.9	I
42	307	17 (D)	1	535.5	I
47	675	32 (K)	1	101.7	III
16	678	31 (K)	2	29.2	III

* D = died; K = killed.
† Genotype of oocysts.

cedures used to concentrate *T. gondii*, some *T. gondii* must be killed because not all mice inoculated with tissues of infected animals become infected. In 20 of 67 instances in the present study, *T. gondii* was found in 20–80% of mice inoculated with homogenates of chicken tissues; the amount of material bioassayed was not the major factor because 20–50% of the entire homogenate from heart and brain of each chicken was used for bioassay. Therefore, it is conceivable that the homogenate from the brain and the heart of chicken no. 230 contained *T. gondii* (bradyzoites) from more than 1 tissue cyst that were of different genotypes.

The number of oocysts shed by 13 cats fed mice infected with 13 isolates is shown in Table I. Cats shed between 19 and 535 million oocysts. Genotyping of material from DNA obtained from oocyst-induced infections in mice indicated that 11 of these isolates were type I and 2 were type III; and mixed infections were not seen (Table I).

Results of the present study confirm our earlier observations that a high proportion of *T. gondii* isolates from chickens from Brazil are of type I (Dubey et al., 2002). In the present study, chickens were from human dwellings in an economically deprived area of Brazil, whereas in the previous study chickens were from rural São Paulo; these localities are approximately 500 km apart. The minimum distance among the houses from where chickens were derived for the present study was 300 m, so the chance of multiple infections from the same source is considered low. Thus, the samples are likely to be independent. With respect to mouse virulence, these *T. gondii* isolates of chickens from Brazil are phenotypically different from those of pigs and chickens from the United States (Dubey, 1995; Dubey et al., 2002; Lehmann et al., 2003); none of the isolates from United States chickens or pigs was virulent for mice. Studies are now in progress to genetically compare isolates of *T. gondii* from free-range chickens from other countries.

In the present study, mice inoculated with tissues of chickens naturally infected with type I isolates survived for at least 8 days. Because tissue cysts are formed in mice as early as 8 days PI, irrespective of the route of inoculation or the stage of *T. gondii* inoculated (Dubey et al., 1998); ingestion by cats of even acutely infected mice in nature is likely to induce oocyst formation. Thus, the likelihood that type I isolates are maintained in nature by oocysts is supported by the results of the present study. Until recently, it was assumed that type I isolates of *T. gondii* do not produce many oocysts. Results of the present study and those reported earlier (Dubey et al., 2002) indicate that type I isolates can induce shedding of large numbers of oocysts in cats. In our opinion, the tissue cyst and oocyst stages are an integral component of the life cycle of *T. gondii*, and there is no evidence at present indicating that either cyst-less or oocyst-less strains of *T. gondii* occur in nature.

Recently, Aspinall et al. (2002) reported astonishing findings that 27 of 71 commercial meat products from grocery stores in Manchester, England, had *T. gondii* DNA and, in all instances, the amplified SAG_2 locus indicated type I isolates. However, their study was based on detecting DNA in ground meat and should not be equated with the present study because, here, viable *T. gondii* was isolated.

Results of experimental infections in rodents indicate that mice and hamsters can be infected simultaneously with more than 1 strain of *T. gondii* (De Roever-Bonnett, 1969; Araujo et al., 1997). However, all strains of *T. gondii* so far isolated from naturally infected animals or humans and genotyped were either 1 of the 3 principal types or a recombinant thereof (Howe and Sibley, 1995; Howe et al., 1997); mixed types have not been documented. Whether these results were partly because of methods of isolation is unknown. Most *T. gondii* isolates recovered from humans were obtained by bioassay in mice or cell culture. For bioassay in mice, 2 or more mice are inoculated with tissue homogenates, and genotype is determined usually from one of the several inoculated mice. In cell culture, some strains might outgrow others and thus the prevailing genotype in vitro may not represent the original isolate. In the present study, DNA was extracted from each of the 5 mice inoculated with tissues of chicken no. 230. We genotyped isolates from all 5 mice because 1 mouse died and 4 had survived. One of the 4 mice that survived was infected with type I; thus, type I *T. gondii* may not be lethal to all mice. Whereas we cannot rule out the possibility of experimental error or contamination, we believe that *T. gondii* of different genotypes found in the same chicken represent a natural multiparasite infection.

LITERATURE CITED

ARAUJO, F., T. SLIFER, AND S. KIM. 1997. Chronic infection with *Toxoplasma gondii* does not prevent acute disease or colonization of the brain with tissue cysts following reinfection with different strains of the parasite. Journal of Parasitology **83:** 521–522.

ASPINALL, T. V., D. MARLEE, J. E. HYDE, AND P. F. G. SIMS. 2002. Prevalence of *Toxoplasma gondii* in commercial meat products as monitored by polymerase chain reaction—food for thought? International Journal for Parasitology **32:** 1193–1199.

DARDÉ, M. L. 1996. Biodiversity in *Toxoplasma gondii*. Current Topics in Microbiology and Immunology **219:** 27–41.

DE ROEVER-BONNET, H. 1969. Congenital *Toxoplasma* infections in mice and hamsters infected with avirulent and virulent strains. Tropical and Geographical Medicine **21:** 443–450.

DUBEY, J. P. 1995. Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. Journal of Parasitology **81:** 410–415.

———, AND C. P. BEATTIE. 1988. Toxoplasmosis of animals and man. CRC Press, Boca Raton, Florida, 220 p.

———, AND G. DESMONTS. 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Veterinary Journal **19:** 337–339.

———, D. H. GRAHAM, C. R. BLACKSTON, T. LEHMANN, S. M. GENNARI, A. M. A. RAGOZO, S. M. NISHI, S. K. SHEN, O. C. H. KWOK, D. E.

HILL, AND P. THULLIEZ. 2002. Biological and genetic characterisation of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*) from São Paulo, Brazil: Unexpected findings. International Journal for Parasitology **32:** 99–105.

———, D. S. LINDSAY, AND C. A. SPEER. 1998. Structure of *Toxoplasma gondii* tachyzoites, bradyzoites and sporozoites, and biology and development of tissue cysts. Clinical Microbiology Reviews **11:** 267–299.

———, P. THULLIEZ, AND E. C. POWELL. 1995. *Toxoplasma gondii* in Iowa sows: Comparison of antibody titers to isolation of *T. gondii* by bioassays in mice and cats. Journal of Parasitology **81:** 48–53.

GRIGG, M. E., S. BONNEFOY, A. B. HEHL, Y. SUZUKI, AND J. C. BOOTHROYD. 2001. Success and virulence in *Toxoplasma* as the result of sexual recombination between two distinct ancestries. Science **294:** 161–165.

———, J. GANATRA, J. C. BOOTHROOYD, AND T. P. MARGOLIS. 2001. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. Journal of Infectious Diseases **184:** 633–639.

HOWE, D. K., AND L. D. SIBLEY. 1995. *Toxoplasma gondii* comprises three clonal lineages: Correlation of parasite genotype with human disease. Journal of Infectious Diseases **172:** 1561–1566.

———, B. C. SUMMERS, AND L. D. SIBLEY. 1996. Acute virulence in mice is associated with markers on chromosome VIII in *Toxoplasma gondii*. Infection and Immunity **64:** 5193–5198.

———, S. HONORÉ, F. DEROUIN, AND L. D. SIBLEY. 1997. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. Journal of Clinical Microbiology **35:** 1411–1414.

JOHNSON, A. M. 1997. Speculation on possible life cycles for the clonal lineages in the genus *Toxoplasma*. Parasitology Today **13:** 393–397.

JUNGERSEN, G., L. JENSEN, M. R. RASK, AND P. LIND. 2002. Non-lethal infection parameters in mice separate sheep type II *Toxoplasma gondii* isolates by virulence. Comparative Immunology, Microbiology and Infectious Diseases **25:** 187–195.

LEHMANN, T., C. R. BLACKSTON, S. F. PARMLEY, J. S. REMINGTON, AND J. P. DUBEY. 2000. Strain typing of *Toxoplasma gondii*: Comparison of antigen-coding and house-keeping genes. Journal of Parasitology **86:** 960–971.

———, D. H. GRAHAM, E. DAHL, C. SREEKUMAR, F. LAUNER, J. L. CORN, H. R. GAMBLE, AND J. P. DUBEY. 2003. Transmission dynamics of *Toxoplasma gondii* on a pig farm. Infection, Genetics and Evolution. **3**: 135–141.

MONDRAGON, R., D. K. HOWE, J. P. DUBEY, AND L. D. SIBLEY. 1998. Genotypic analysis of *Toxoplasma gondii* isolates from pigs. Journal of Parasitology **84:** 639–641.

OWEN, M. R., AND A. J. TREES. 1999. Genotyping of *Toxoplasma gondii* associated with abortion in sheep. Journal of Parasitology **85:** 382–384.

RUIZ, A., AND J. K. FRENKEL. 1980. Intermediate and transport hosts of *Toxoplasma gondii* in Costa Rica. American Journal of Tropical Medicine and Hygiene **29:** 1161–1166.

SILVA, D. S. S., L. M. G. BAHIA-OLIVEIRA, S. K. SHEN, O. C. H. KWOK, T. LEHMANN, AND J. P. DUBEY. 2003. Prevalence of *Toxoplasma gondii* in chickens from an area in southern Brazil highly endemic to humans. Journal of Parasitology **89:** 394–396.

SU, C., D. K. HOWE, J. P. DUBEY, J. W. AJIOKA, AND L. D. SIBLEY. 2002. Identification of quantitative trait loci controlling acute virulence in *Toxoplasma gondii*. Proceedings of the National Academy of Sciences of the United States of America **99:** 10753–10758.

J. Parasitol., 89(4), 2003, pp. 853–855

Effect of Vaccination with a Recombinant Fusion Protein Encoding an Astacinlike Metalloprotease (MTP-1) Secreted by Host-Stimulated *Ancylostoma caninum* Third-Stage Infective Larvae

Peter J. Hotez, James Ashcom, Bin Zhan, Jeffrey Bethony, Alex Loukas, John Hawdon, Yang Wang, Qun Jin, Karen C. Jones, Azra Dobardzic, Reshad Dobardzic, Janelle Bolden, Idong Essiet, Walter Brandt, Philip K. Russell, Bernard C. Zook*, Brian Howard†, and Marco Chacon†, Department of Microbiology and Tropical Medicine and Sabin Vaccine Institute, The George Washington University Medical Center, 2300 Eye Street NW, Washington, D.C. 20037; *Department of Pathology, The George Washington University Medical Center, 2300 Eye Street NW, Washington, D.C. 20037; †Paragon Bioservices, Inc., Johns Hopkins Bayview Research Campus, Alpha Center, 5210 Eastern Avenue, Baltimore, Maryland 21224. *e-mail: mtmpjh@gwumc.edu*

ABSTRACT: Laboratory dogs were vaccinated intramuscularly with a recombinant fusion protein (expressed and isolated from *Escherichia coli*) formulated with the Glaxo SmithKline Adjuvant System 02 (AS02). The fusion protein encoded Ac-MTP-1, a developmentally regulated astacinlike metalloprotease secreted by host-stimulated *Ancylostoma caninum* third-stage larvae (L3). Control dogs were injected intramuscularly with an equivalent amount of AS02 adjuvant alone. The vaccinated and control dogs were then challenged by s.c. injection of 500 L3 of the canine hookworm *A. caninum*. The vaccinated dogs developed prechallenge immunoglobulin G2 (IgG2) antibody responses specific to anti–Ac-MTP-1-fusion protein with titers ranging between 1:40,000 and 1:364,000, whereas they developed antigen-specific immunoglobulin E antibody responses with titers ranging between 1:500 and 1:1,500. By immunoblotting, canine sera obtained from the vaccinated dogs recognized a protein of the estimated apparent molecular weight of Ac-MTP-1 in activated L3 secretory products. Spearman rank order correlations between the canine intestinal adult hookworm burden and quantitative egg counts at necropsy and anti-Ac-MTP-1 IgG2 antibody titers revealed a statistically significant inverse association ($r = -0.89$; $P = 0.04$), suggesting that this molecule offers promise as a recombinant vaccine.

Infective third-stage *Ancylostoma* hookworm larvae (L3) release a zinc-dependent metalloprotease that migrates with an apparent molecular weight (MW) of 50 kDa (Hawdon et al., 1995). The enzyme is released specifically in response to stimuli that induce feeding and development in the L3 (Hawdon et al., 1995) and probably functions in either parasite skin and tissue invasion or ecdysis (Hotez et al., 1990). Serum from hookworm-infected patients in China was used as an immunoscreening probe to isolate complementary DNA (*Ac-mtp-1*) from an *A. caninum* L3 expression library that encodes a 547 amino acid (MW 61,730) zinc metalloprotease of the astacin family (Zhan et al., 2002). Astacins (named for a digestive protease from the crayfish *Astacus astacus*) are characterized by a propeptide, a catalytic domain containing the characteristic zinc-binding region, and a 'Met turn.' In addition, Ac-MTP-1, like some members of this family, contains signature epidermal growth factor and CUB domains (Zhan et al., 2002). Ac-MTP-1 is structurally unrelated to Ac-MEP-1, an adult hookworm gut luminal metalloprotease of the neprilysin family (Jones and Hotez, 2002). The processed form of Ac-MTP-1 represents the molecule responsible for the proteolytic activity found in host-stimulated L3 secretory products (Zhan et al., 2002).

Because of its role in parasite-derived tissue invasion and molting,